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Original Research

Production of gold nanoparticles by *Streptomyces djakartensis* isolate B-5

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Abstract

Objective(s): Biosynthesis of gold nanoparticles (NGPs) is environmentally safer than chemical and physical procedures. This method requires no use of toxic solvents and synthesis of dangerous products and is environmentally safe. In this study, we report the biosynthesis of NGPs using *Streptomyces djakartensis* isolate B-5.

Materials and Methods: NGPs were biosynthesized by reducing aqueous gold chloride solution *via* a *Streptomyces* isolate without the need for any additive for protecting nanoparticles from aggregation. We characterized the responsible *Streptomycete*; its genome DNA was isolated, purified and 16S rRNA was amplified by PCR. The amplified isolate was sequenced; using the BLAST search tool from NCBI, the microorganism was identified to species level.

Results: Treating chloroauric acid solutions with this bacterium resulted in reduction of gold ions and formation of stable NGPs. TEM and SEM electro micrographs of NGPs indicated size range from 2- 25 nm with average of 9.09 nm produced intracellular by the bacterium. SEM electro micrographs revealed morphology of spores and mycelia. The amplified PCR fragment of 16S rRNA gene was cloned and sequenced from both sides; it consisted of 741 nucleotides. According to NCBI GenBank, the bacterium had 97.1% homology with *Streptomyces djakartensis* strain RT-49. The GenBank accession number for partial 16S rRNA gene was recorded as JX162550.

Conclusion: Optimized application of such findings may create applications of *Streptomycetes* for use as bio-factories in eco-friendly production of NGPs to serve in demanding industries and related biomedical areas. Research in this area should also focus on the unlocking the full mechanism of NGPs biosynthesis by *Streptomycetes*.

Keywords: Bio-factory, Green synthesis, Nanogold, Biosynthesis, rRNA, *Streptomyces*

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Introduction

Metallic nanoparticles are presently applied in different fields of electronics, biotechnology, cosmetics and medicine (1, 2). Biomedical applications of nanogold particles [NGPs] have received great attention in recent years (3, 4, 5) and have attracted enormous scientific and technological interest due to their unique properties (6, 7). In the recent years, scientists accelerate the application of nanoparticles to the prevention, biodiagnosis (3), drug delivery (8, 9) and treatment of cancer (10-12). Nanotechnology represents economic alternatives for chemical and physical methods of nanopaticles formation. It combines biological principles with physical and chemical procedures to generate nano-sized particles with specific functions. Among the biological organisms, some microorganisms such as bacteria, fungi, and yeasts have been exploited for biosynthesis of nanoparticles (13, 14). Among tested microorganisms, species of Streptomyces have the most diversity for production of intracellular NGPs (15); however, they are becoming promising BioFactories in this regard (16). Various methods have been reported over the last two decades for the synthesis of Au nanoparticles, who involve reduction of $HAuCl_4$ with a chemical reducing agent or some organic compounds (17); however, green techniques in the biosynthesis of NGPs would benefit from the development of clean, nontoxic, and environmentally acceptable procedures concerning micro-organisms. *Streptomycetes* are grampositive aerobic bacteria; some of them are capable of transformation of some heavy metals ions and reducing them to amorphous or crystalline metal particles. In this paper we report the biosynthesis of NGPs by reducing aqueous gold chloride solution *via* a *Streptomyces* isolate without the need for any additive for protecting nanoparticles from aggregation. We characterized the responsible *Streptomycete*; its genome DNA was isolated, purified and 16S rRNA was amplified by PCR. The amplified isolate was sequenced; using the BLAST search tool from NCBI, the microorganism was identified to species level.

Materials and Methods

Soil sampling and isolation of Streptomyce-tes

Soil samples were randomly collected from grasslands, orchards and vegetable fields in Sefteh village in Kerman Province of Iran. Soil samples were taken from a depth of 10 to 20 cm below the soil surface using an open-end soil borer (20 cm in depth, 2.5 cm in diameter) as described by Lee and Hwang (18). After air-drying at room temperature for 7 to 10 days, soil samples were sieved with a 0.8 mm mesh and preserved in polyethylene bags at room temperature. Ten gram of each soil sample was brought up to final volume of 100 ml by adding sterile distilled water. The mixtures were shaken vigorously for one hour and allowed to settle for an extra hour. The supernatant was gently removed and diluted to 10^{-2} - 10^{-6} with sterile distilled water. Aliquots of each of 10^{-3}- 10^{-6} dilutions added to autoclaved CGA at 50° C (1:20 v/v) and poured to sterile Petri plates. After solidification, plates incubated at 28° C for 7- 10 days. From day 7, *Streptomyces* colonies isolated as pure cultures on CGA, grown at 28° C for one week and stored at 4° C for further investigations.

Culture medium

Casein glycerol agar (CGA) was prepared from basic ingredients as described by Dhingra and Sinclair (19) and used as *Streptomyces* growth medium.
Excluding the agar from CGA, casein glycerol (CG) medium was applied for *Streptomyces* submerged cultures.

Submerged cultures and preparation of biomass

CG media used to prepare liquid media. Isolated *Streptomyces* inoculated to CG

media and shaked on rotary shakers at 29° C and 130 rpm for five days; cultures filtered through Whatman filter paper No. 1, rinsed twice with distilled water to remove the media. The resulting biomass was then used for screening.

Screening for production of NGPs

Portions of biomass of each isolate removed and divided to two samples in glass tubes of bench centrifuge. Both samples centrifuged at 4000 rpm/5 min, clear supernatants discarded and colorful biomass resuspended in DDH_2O and subjected to two more cycles of low centrifugations. Two ml of 10^{-3} mol of $HAuCl_4$ added to 0.2 g of biomass pellet and agitated to uniformly resuspend; while control received plain DDH_2O. Both samples placed on rotary shaker at 130 rpm and 29° C for 12 – 24 hr. Color change from yellow to purple red was indicative of bioactivity of tested *Streptomyces* in production of NGPs; however, NGPs were confirmed with further analysis as described in coming sections.

Verification of intra-cellular and/or extra-cellular production of NGPs

To verify whether NGPs are produced intra and/or extra-cellular, portion of colored biomass (treated for 12-24 hr with $HAuCl_4$) was subjected to low speed centrifugation at 4000 rpm for 10 min. Since the supernatant was colorless and clear, it was not further processed for presence of NGPs (all particle sizes of NGPs are colorful as red, purple, violet and not yellowish). The red violet pellet was resuspended in DDH_2O and subjected to two more cycles of low speed centrifugation. The final pellet resuspended in DDH_2O and applied to 0.2 μ filter. The filtrate was colorless clear but the retained biomass was red violet indicative of probable production of intra-cellular presence of NGPs. The Millipore filter-retained biomass relatively desiccated under vacuum and used for further TEM and Flame photometric analysis.

TEM studies

Streptomyces djakartensis isolate B-5 selected through screening as described at previous section and used for evaluations with TEM (Philips CM10, 100kv).

The biomasses of both treated and control were investigated as described by Mandal, *et al.* (20). Carbon and formvar coated TEM grids were applied with a thin layer from 12 – 24 hr $HAuCl_4$ treated mycelial suspension (in DDH_2O) and excess removed by Whatman filter paper No 1. Electro micrographs with scale bars prepared from more than 200 GNPs and used to measure particle size distribution and mean size values; however, no Zetasizer was available to do this task with higher precision.

Flame photometric analysis

The Millipore filter-retained biomass of *S. djakartensis* isolate B-5 having red violet color was further rinsed with large volume of DDH_2O to retain the mycelia and spores but wash off traces of ionic Au from the biomass. Filter-retained biomass (0.2 g, relatively dry) was suspended in 20 ml DDH_2O and sonicated using a sonicator (NSK dental sonicator, Varios 570 Ipiezo engine) to rapture the cells, homogenize the sample and release intracellular NGPs. From homogenized sample (dilution of 10^{-1}), further dilutions of 10^{-4}, 10^{-5} and 10^{-6} made in DDH_2O and used to measure NGP concentration by a flame photometer (Varian AA240FS) equipped with gold lamp. Average ppm concentration revealed by the instrument used to calculate the approximate conversion yield (Au ion to NGPs).

Uv–vis spectroscopy

The produced NGPs also analyzed by use of *uv–vis* spectroscopy. In this regard, the sample which was used in flame

photometric analysis (previous section) was also used for this evaluation. The spectrum was taken from 400-800 nm wave lengths with a double beam spectrophotometer Model ScanDrop 250-211F075.

STM studies
The criteria of spore surface and myclial morphology determined for this *Strepyomyces* isolate by a STM (Lutz 100A, 20kv) as described by Gehring, *et al.,* (21).

Extraction of Genomic DNA
The active *Streptomyces* sp. isolate (earlier designated as B-5, while isolated) was propagated in submerged culture of CG medium in a shaker incubator at 28°C and 130 rpm for 6 days.
The colonies were then harvested using bench centrifugation at 10000 rpm for 5 min and genomic DNA isolated by CTAB method as described by Baharlouei, *et al.* (22).

Polymerase chain reaction (PCR)
Two sets of primers consist of F16 SS (5'-ACGGGTGAGTAACACG-3') and R16 SS (5'-TACCGCGGCTGCTGGCACG-3') were designed based on the known sequences from the family 16S rRNA genes of *Streptomyces* spp. The primers were synthesized in Isogene Company, Netherlands. A PCR was run which utilized
melting temperature of 94°C for 5 min, following 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and final extension at 72°C for 7 min. Electrophoresis of sample in 1% agarose gel and photography of resolved band performed as described by Sambrook and Russell (23).

Cloning of DNA fragment of 16S rRNA gene of Strepyomyces isolate B-5
The amplified genes fragment was purified with an AccuPrep® PCR purification kit (Takapouzist, Iran), ligated into the pTZ57R/T vector using InsT/A Clone™ PCR cloning kit (Fermentas, Germany). Escherichia coli, XL1blue and plasmid pTZ57R/T were provided from Cinagen Co., Iran and Fermentas Co., Germany, as bacterial host strain and vector in cloning experiments, respectively.

Results
Soil sampling and isolation of Streptomyces spp
From the soil samples cultured, over 50 *Streptomyces* isolated recovered which used for further evaluations.

Screening for production of NGPs
The isolate, designated as B-5, showed high performance in production of NGPs compared to others.
The isolate was further characterized and identified as *Streptomyces djakartensis*. As indicated in Figure 1, color change from pale yellow (Au ion) to red violet (production of NGPs) was indicative of NGPs production by this isolate.

NGPs size analyses
To assess approximate size properties of NGPs for *S. djakartensis* isolate B-5, by

Figure 1. Color change indicative of NGPs production by Streptomyces strain; the color change of aqueous solution of HauCl$_4$ to red after 24 h incubation with active biomass of this strain was indicative of NGPs production. Test tube containing before (right) and after (left) 24 h incubation of *Streptomyces djakartensis* isolate B-5.

use of TEM electro micrographs, NGP sizes measured, particle size distribution plotted, mean value and standard deviation
determined statistically using Microsoft Office Excel 2007 software.

Analysis of particle size distribution and mean of particle sizes are presented in Fig. 2.

As indicated in the figure, intracellular NGPs produced by S. *djakartensis* isolate B-5 range from 2- 25 nm with mean size of 9.09 nm.

These values assessed from evaluating 213 NGPs in electro micrographs.

As indicated earlier, no Zetasizer was available to measure particle sizes with higher precision.
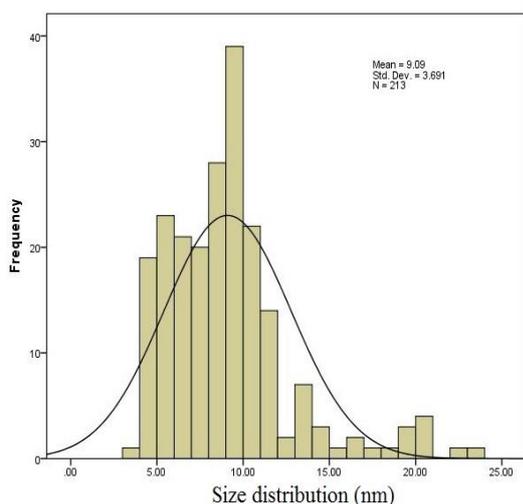
Figure 2. NGPs particle size distribution and mean of particle sizes produced by *Streptomyces djakartensis* isolate B-5.

TEM studies

The NGPs were observed within the cells in TEM electro micrographs.

NGPs production by *S. djakartensis* isolate B-5 is indicated in Figure 3.

The approximate sizes of NGPs were estimated 2- 25 nm. Presence of both spherical (probably icosahedra) and triangular NGPs within the cells were noticeable; however, spherical ones were dominant. Although some aggregations were noticeable in the electro micrographs, but magnification

of the pictures clearly revealed margins of NGPs in each aggregated mass.
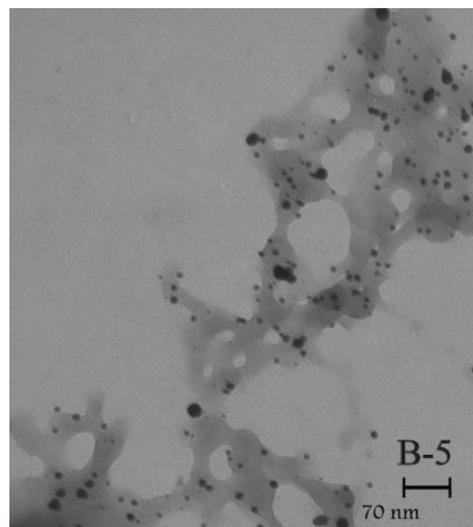
Figure 3. TEM electro micrograph of intracellular production of NGP by *Streptomyces djakartensis* isolate B-5. The approximate sizes of NGPs are 2- 25 nm. Both spherical (probably icosahedra) and triangular NGPs are noticeable. Some aggregations are also present which upon magnification margins of NGPs is recognizable in each aggregated mass.

Flame photometric analysis

The result of flame photometric analysis of sonicated biomass of *S. djakartensis* isolate B-5 (red violet biomass retained on 0.2μ Millipore filter) indicated presence of gold in the dilutions used. The average mean concentration of gold from the three dilutions (10^{-4}, 10^{-5} and 10^{-6}) was indicative of 12.93 ppm.

Considering the original used concentration of Au ion in 10^{-3} mol as 197 ppm, the percentage of recovery calculated was 6.56%. The low recovery is due to use of low ratio of original biomass to Au ion in the experiment; however the recovery would improve if the proportions are optimized.

Uv–vis Spectroscopy:
The *uv–vis* spectrum of NGPs produced by *S. djakartensis* isolate B-5 indicated presence of a broad single peak with maximum absorbance at 530 nm. The spectrum is indicated in Figure 4.

STM studies

STM electro micrograph of *Streptomyces djakartensis* isolate B-5 was indicative that spores form in simple chains and have smooth surfaces.
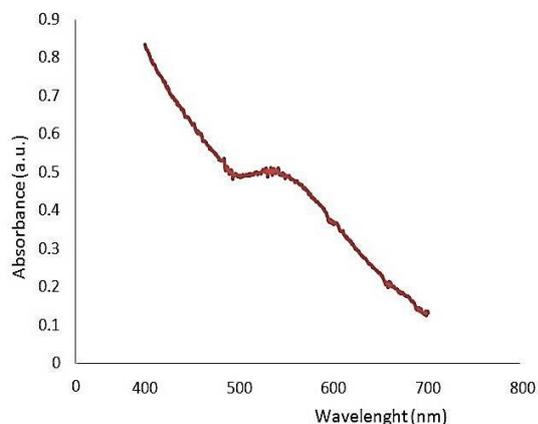
Figure 4. UV-Vis Spectrum of nanogold particles produced by *Streptomyces djakartensis* isolate B-5. Presence of a broad single peak with maximum absorbance at 530 nm is prominent.

No specific sculptures detected on spore surfaces. STM electro micrograph of this isolate is indicated in Figure 5. As indicated in the figure, this strain forms long straight chains of spores with smooth surfaces.

Cloning of 16S rRNA genes

Fragment of 16S rRNA gene was cloned as of ~800 bp from *Streptomyces* isolate B-5 *via* forward and reverse primers of 16 SS using cDNA as the template.
The amplified PCR fragment was cloned and sequenced from both sides.
The fragment consisted of 741 nucleotides. The result of amplification of 16S rRNA gene of *Streptomyces* isolate B-5 is indicated in Figure 6.

DNA sequencing

Recombinant plasmid was sequenced in both directions by extending 16 SS reverse and forward primers by Automatic DNA Sequencer (Macrogene, Korea).
The sequence data of *Strepyomyces* isolate B-5 was analyzed using the Chromas software version 1.41.

Nucleotide fragment BLAST-ed by nucleotide blast program in the NCBI databank and sequence aligned with related sequences from other isolates of *Streptomyces* by DNAMAN software package (Lynnon Biosoft, Quebec, Canada).
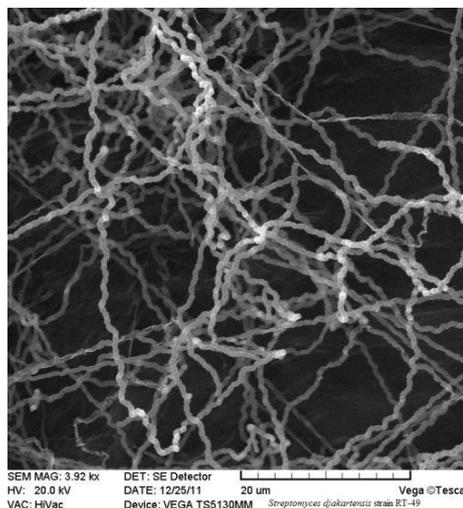
Figure 5. STM image of *Streptomyces djakartensis* isolate B-5. This strain forms long straight chains of spores with smooth surfaces.
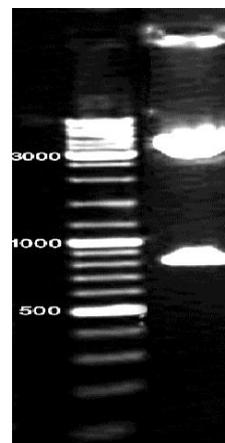
Figure 6. Amplification of 16S rRNA gene (800 bp) of *Streptomyces* isolate B-5 (right) from recombinant pTZ57R/T plasmid (3000 bp, digested with EcoRI and PstI enzymes) along the DNA size ladder (left, 100 bp).

Nucleotide sequence homology matrix and accession number

Homology matrix of the sequence of partial 16S rRNA gene from *Streptomyces* isolate B-5, and sequences of other families of partial 16S rRNA genes are represented in Table 1.

Table 1. Homology matrix of the sequence of partial 16S rRNA gene from *Streptomyces* isolate B-5 and partial sequences of related species.

B-5: *Streptomyces djakartensis* isolate B-5

Homology matrix of 11 sequences

sequence of B5	100%									
Streptomyces djakartensis strain RT-49	97.1%	100%								
Streptomyces enissocaesilis strain RT-46	97.1%	100.0%	100%							
Streptomyces geysiriensis strain RT-63	97.1%	100.0%	100.0%	100%						
Streptomyces olivaceus strain RT-54	97.1%	100.0%	100.0%	100.0%	100%					
Streptomyces plicatus strain RT-57	97.1%	100.0%	100.0%	100.0%	100.0%	100%				
Streptomyces rochei strain T170	97.1%	99.6%	99.6%	99.6%	99.6%	99.6%	100%			
Streptomyces rochei strain Lac3	97.1%	99.5%	99.5%	99.5%	99.5%	99.5%	99.4%	100%		
Streptomyces avidinii strain SU4	97.1%	98.2%	98.2%	98.2%	98.2%	98.2%	99.3%	99.3%	100%	
Rhodococcus sp. SH05-06	87.9%	89.5%	89.5%	89.5%	89.5%	89.5%	88.9%	88.7%	88.6%	100%
Thermomonospora sp. YIM 75085	84.7%	84.4%	84.4%	84.3%	84.4%	84.4%	85.5%	85.7%	83.3%	82.7%	100%

As indicated in the table, *Streptomyces* isolate B-5 has 97.1% homology with *Streptomyces djakartensis*. The GenBank accession number for partial 16S rRNA gene of *Streptomyces* isolate B-5 was recorded as JX162550.

Discussion

The area of microbial biosynthesis of NGPs is a relatively new. The mechanism of biosynthesis has not been fully elucidated in the literature; however, according to Kitching (24) Au^{+3} ions switch on oxidative stress response genes. Possible methylation step reduces Au^{+3} to Au^{+1}, then NADH (or NADPH) dependant proteins (e.g. ATPase, 3-glucan binding protein and glycer-aldehydes 3phosphate dehydrogenase) in the cell wall and cytoplasmic regions reduce the Au^{+1} to Au^0.

The neutral Au forms NGPs, which in turn are stabilized by proteins, most likely different from the proteins involved in the synthesis.

Streptomycetes are characterized by complex secondary metabolites [3]. These diverse characteristics plus their simple growth needs has made them to receive high attention in screening for NGPs among microorganisms; however, they produce over two-thirds of the clinically useful antibiotics of natural origin (25). Colony morphology, SEM electro micrographs and sequencing of 16sRNA of our tested microorganism indicated that it belongs to the genus *Streptomyces*. Its genome DNA was isolated, purified and 16S rRNA was amplified by PCR. The amplified isolate was sequenced; using the BLAST search tool from NCBI the microorganism was identified. *Streptomyces* isolate B-5 had 97.1% homology with *S. djakartensis*. Since it is the first time that its ability in production of gold nanoparticles is investigated, it was not feasible to deduce NGPs metabolic pathways from the genetic analysis and even phylogenic tree of their closely related species. *Streptomyces djakartensis* isolate B-5 examined in this study proved its ability to produce gold nanoparticles from an aqueous solution containing $HAuCl_4$.

The results clearly showed that NGPs formed by reduction of Au (III) ions by the bacterium. Evaluations of the electro

micrograph of intracellular biosynthesized NGPs indicated that they ranged in size from 2 to 25 nm with average size of 9.09 nm.

The results of our investigation and similar studies may create valuable applications of *Streptomycetes* as bio-factories for eco-friendly production of NGPs to serve in demanding industries and relative biomedical areas. For optimization, research in this area should focus on unlocking the full mechanism of NGPs biosynthesis by *S. djakartensis* isolate B-5. We would like to postulate that biological synthesis of NGPs is worth because of eco-friendly, environ-mentally acceptable solvent system, and lack of toxic chemicals involved in this green biosynthesis method; however more resea-rch is needed to optimize the procedure.

Conclusion

Optimized application of such findings may create applications of *Streptomycetes* for use as bio-factories in eco-friendly production of NGPs to serve in demanding industries and related biomedical areas. Research in this area should also focus on the unlocking the full mechanism of NGPs biosynthesis by *Streptomycetes*.

Acknowledgements

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